REMARKS

Entry of the above amendments and reconsideration of this application is respectfully requested. Claims 1-94 have been cancelled. Claim 95 has been amended to include steroid hormone depleted serum. Support for such an amendment can be found generally throughout the application including, for example, Example 2, and paragraph [0724]. Thus, no new matter has been added by these amendments. In view of the amendments and the following remarks, it is believed that all rejections are overcome and that this application is in condition for allowance.

Claim Objections

Claims 105-109 have been objected to as being dependent upon a rejected base claim. The Examiner suggests that these claims would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims. Briefly, the allowable subject matter includes claim 95 with the exception of cell lines T47D and ZR-75-1. Applicants thank the Examiner for the indication of allowable subject matter; however, it is hereby submitted that the non-allowable subject matter is also patentable over the references cited by the Examiner for at least the reasons set forth below.

Claim Rejections under 35 U.S.C. §103(a)

Claims 95-104 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Furuya et al (Cancer Research, December 1989, Vol. 49, pp 6670-6674) in view of Hoffman ('The Biochemistry of Clinical Medicine', 1970, pages 48-55). The Examiner contends that Furuya et al. teach that a bovine serum albumin fraction containing globulin remnants inhibited cell growth but that globulin-free bovine serum albumin did not inhibit cell growth. The Examiner then reasons that one skilled in the art would reasonably conclude that serum globulins were the cause of the growth inhibition. The Examiner also contends that Furuya et al. teach that estradiol can overcome this inhibition. The Examiner acknowledges that Furuya et al. fail to teach growth stimulation by estrogen in the presence of IgA or IgM but suggests that Hoffman et al. provides evidence that these components are present within the globulin fraction of serum. The Examiner therefore reasons that one skilled in the art would be motivated to test the inhibitory contributions of serum globulin proteins to identify the factor(s) responsible for growth inhibition. Applicants respectfully disagree.

Furuya et al. teach the use of a globulin free serum albumin to look at the direct effects of estradiol and Tamoxifen on MCF-7 cells. Furuya et al. teach against the use of a bovine serum albumin V medium for this purpose because it contains globulin remnants, which Furuya et al. suggests has at least some growth inhibitory properties. Thus, Furuya et al. are looking at the effects of estradiol and Tamoxifen rather than any specific components that can be purified for the purpose of inhibiting cancer cell growth. Additionally, although the cells lines T47D and ZR-75-1 are mentioned by Furuya et al., nowhere do they teach or suggest growth inhibition of these cell lines with IgA and/or IgM as presently described and claimed. Indeed, T47D and ZR-75-1 are referred to by Furuya et al. merely to indicate that they are established human breast cancer cell lines that can be "used to study the mechanisms of action of exogenous estrogens and antiestrogens on cell proliferation in various culture media." See Furuya et al. at page 6670, second sentence under Introduction. ZR-75-1 is also mentioned as showing "direct E_2 stimulation of growth and TAM inhibition, even in serumless media." See Furuya et al. at page 6670 top of right hand column. Furuya et al. also reference that Soto et al. "have indicated that DC-treated human serum and FBS contain a specific growth inhibitor for E_2-responsive T47D and MCF-7 cells and E_2 neutralizes it resulting in indirect promotion of cell growth." See Furuya et al. at page 6670 top of right hand column. These few citations are also referenced in the Discussion section (see page 6673) and are the only mention of T47D and ZR-75-1 cell lines. Furuya et al. only used MCF-7 and HBC-4 cells to conduct the experiments described therein. Thus, Furuya et al. fail to teach or suggest any growth inhibition of T47D and ZR-75-1 cells by any of the experimental methods performed.

Turning now to Hoffman et al., this reference merely shows the distribution of IgG, IgA, IgM and IgD in normal serum. Even assuming, *arguendo*, that the normal serum depicted in Fig. 4 of Hoffman et al. is the same as the bovine serum albumin V fraction used by Furuya et al., there is no teaching or suggestion that these immunoglobulins, and specifically IgA and IgM as presently claimed, are responsible for the growth inhibition suggested by Furuya et al. Indeed, BSA-V is generally known in the art as being a very crude fraction containing hundreds of serum proteins and many other impurities. One skilled in the art could not ascertain that any growth inhibitory function was the result of IgA and IgM prior to the Applicants' discovery. No

specificity can be obtained by using crude fractions. For any growth inhibitor identification to be accomplished, these inhibitors must be purified and further tested to show they are responsible for cell growth inhibition. These immunoglobulins, through many purification steps, were eventually shown to be responsible for growth inhibition of cancer cells. See the present application at Example 17. Such an association was not possible prior to these experimental results.

In addition to the above, Applicants note that Furuya et al. was published in 1989 and Hoffman et al. was published as early as 1954 (more recently in 1970). If the identification of IgA and IgM was an obvious undertaking as suggested by the Examiner, Applicants submit that such a discovery would have taken a much shorter period of time. As discussed in Example 17, prior documents have suggested a serum-borne growth inhibiting factor(s) but had yet to identify its existence. More specifically, Soto et al. (*J. Steroid Biochem. Molec. Biol.* Vol. 43, No. 7, pp. 703-712 (1992)) attempted such an undertaking and published their unsuccessful results in 1992. Thus, almost three full years after the Furuya et al. publication, those skilled in the art were unable to purify these serum-borne growth inhibiting factor(s). In fact, Soto et al. presents evidence which would lead one skilled in the art away from IgA and IgM as the growth inhibiting factors. Soto et al. state that "[e]strocolyone-I activity seems to be due to a protein of an apparent native M_w of 70-80 kDa..." See Soto et al. at page 703, Summary, last full sentence. It is well known in the art that IgA (dimer) has a molecular weight of 320kDa and IgM has a molecular weight of 900kDa. Moreover, Soto et al. state that "[f]urther purification of estrocolyone-I is being pursued, a step necessary for a mechanismtic understanding of its action and that of estrogens on the control of cell proliferation." See Soto et al. at page 711 last sentence. Given the above, the Soto et al. reference is being filed concurrently herewith as evidence showing the failure of others in achieving the presently claimed methods.

Conclusion

In view of the foregoing, it is submitted that all objections and rejections have been overcome. Withdrawal of the objections and rejections and allowance of the claims are solicited.

Request for Interview

In the event that the Examiner finds any reason that the application cannot be allowed in its present form, the Applicant wishes to conduct an interview with the Examiner prior to any further action in order to provide an opportunity for coming to agreement upon allowable claims. To arrange the interview, the Examiner should call the undersigned attorney at the telephone number given.

Respectfully submitted,

June 7, 2010

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A PLASMA-BORNE SPECIFIC INHIBITOR OF THE PROLIFERATION OF HUMAN ESTROGEN-SENSITIVE BREAST TUMOR CELLS (ESTROCOLYONE-I)

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Summary—Charcoal-dextran stripped serum/plasma supplemented media specifically inhibit the proliferation of estrogen-sensitive cells in culture conditions; estrogens cancel this effect. Here, we further characterize this phenomenon using human estrogen-sensitive breast cancer MCF_7 cells and human serum/plasma. The serum/plasma-borne inhibitory activity (estrocolyone-I) is a non-dialyzable, heat-stable (60°C × 2 h), protease-sensitive macromolecule and it is not extractable by organic solvents. Estrocolyone-I activity is retained after dialysis against 6 M urea or 10–100 mM dithiothreitol; however, simultaneous treatment with 6 M urea and 10–100 mM dithiothreitol completely abolishes its inhibitory activity. The inhibitory effect of serum is not due to serum albumin, nor to estrogen trapping by albumin or by sex hormone-binding globulin. Substantial purification was achieved by a combination of chromatographic techniques (dye-affinity, ion exchange, hydrophobic interaction chromatography). Estrocolyone-I activity seems to be due to a protein of an apparent native M_r of 70–80 kDa and an isoelectric point of 4.5–4.8.

INTRODUCTION

The regulation of cell proliferation by estrogens is incompletely understood. This subject is currently being explored under three different working hypotheses. The direct-positive hypothesis that proposes that estrogens *per se* trigger the multiplication of their target cells [1–4]. Alternatively, the indirect-positive hypothesis that proposes that estrogens induce the synthesis and/or release of growth factors [5] which, in turn, cause proliferation of their target organs. The autocrine mechanism [6], a variation of this latter hypothesis, proposes that estrogens would induce the synthesis and secretion of a variety of growth factors by their own target cells. In turn, these autocrine growth factors would elicit the proliferation of the same cells which secreted them [7, 8]. In contrast to predictions of the positive hypotheses, several reports showed that cells that are estrogen-dependent when inoculated into animals proliferate maximally in serumless medium regardless of the presence of estrogen [9–13]. Because of these data, we have been engaged in exploring the indirect-negative hypothesis: it proposes that estrogens neutralize the action of blood-borne specific proliferation inhibitor(s) [10, 11, 14–16].

MCF_7 cells were chosen as a model because (a) they grow as estrogen-dependent tumors in athymic mice [17–19], (b) charcoal-dextran stripped human serum inhibits their proliferation in a dose-dependent pattern [10, 11], and (c) estrogens cancel the inhibitory effect of serum at physiological concentrations. Because these cells also proliferate maximally in serumless medium regardless of the presence of estrogens, we concluded that serum contains a specific inhibitor of the proliferation of estrogen-sensitive cells, estrocolyone-I; estrogens would merely neutralize this inhibitory effect [10, 11, 16]. Here, we describe the characterization and partial purification of estrocolyone-I.

METHODS

Cell culture procedures

A clonal population of the human breast cancer MCF_7 cell line was used in these experiments [10]. To assess the inhibitory effect of serum/plasma or partially purified serum/plasma fractions, 5×10^4 cells were seeded in Costar 3512 Multiplates in 5% fetal bovine

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serum (FBS); they were allowed to attach for 24 h. Then, 1 ml of Dulbecco's modification of Eagle medium (DME) plus 2 μg transferrin (T)/ml and 100 ng insulin (I)/ml (IT-DME) alone or IT-DME plus (i) charcoal-dextran stripped (CD) serum or (ii) partially purified preparations were placed in each well. Each preparation was tested by triplicate in the presence and absence of 30 pM 17β-estradiol (E_2). Phenol-red free media were used in all experiments [20]. At selected time intervals cells were lysed [10% Zapoglobin (Coulter Electronics, Hialeah, FL)] and nuclei counted on a Coulter Counter Model Z_{f}. Previous to testing the inhibitory potency of partially purified estrocolyone-I, fractions were concentrated by ultrafiltration (YM30 Amicon membranes, nominal cutoff = 30 kDa) and dialyzed through cellulose membranes (nominal cutoff = 25 kDa) against a buffer suitable for tissue culture (TC buffer: 100 mM NaCl, 25 mM HEPES, pH 7.4). Estrogens were removed from serum and from partially purified plasma fractions by charcoal-dextran stripping [10, 11].

Purification procedures

Plasma coagulation factors were removed by precipitation with 80 mM $BaCl_2$ [21]. Matrex-green chromatography (Amicon Corp., Lexington, MA) of $BaCl_2$-treated plasma was used to remove human serum albumin (HSA). The column was equilibrated with 25 mM bistrispropane (BTP), pH 7.0, and 0.6 ml plasma were loaded/ml packed gel. The breakthrough was used for estrocolyone-I purification and the bound fraction was eluted with 2 M NaCl, 25 mM BTP, pH 7.0. This fraction was used in protease digestion experiments. For anion exchange chromatography, start buffers were 50 mM piperazine pH 6.0 and BTP, pH 7.0 and 9.5. Limit buffers contained 1 M NaCl. 50 to 200 mg Matrex-green breakthrough protein were injected into a FPLC MonoQ Hr10/10 column equilibrated with start buffer; separation was carried out by linear gradient elution. For hydrophobic interaction chromatography, samples were equilibrated in start buffer, injected into a phenyl-Superose HR5/5 column and resolved by linear gradient [50 mM sodium phosphate buffer containing 0.4 to 2M $(NH_4)_2SO_4$ as start buffer and 20% acetonitrile-50 mM Na phosphate pH 7.0 as limit buffer]. To determine the pI of estrocolyone-I, chromatofocusing was performed in a MonoP HR25/5 column; pH gradients from 7.0 to 4.0 and from 5.5 to 4.0 were generated with 10% Polybuffer 74. Separation by gel filtration in FPLC Superose 12 HR 16/50 (bed volume: 98 ml) and in Superdex 200 XK 26/60 (bed volume: 320 ml) columns were used for assessing the native molecular weight of estrocolyone-I. Molecular weight standards were used to monitor the elution pattern. FPLC instruments and reagents were from Pharmacia-LKB (Piscataway, NJ). All buffers contained 1 mM benzamidine, a protease inhibitor.

Protease hydrolysis

Charcoal-dextran stripped rooster serum was incubated at 37°C for 2 h with and without 0.25 mg trypsin/ml (8000 BAEE U/mg). 5000 U soybean trypsin inhibitor/ml were added to stop the proteolytic effect; after dilution to 10% with IT-DME, sera were tested for estrocolyone-I activity. Proteases were removed from human plasma by Matrex-green chromatography; aliquots were dialyzed against 50 mM $CaCl_2$–50 mM Tris–HCl, pH 8.0 for alpha-chymotrypsin and against 1 mM EDTA, 1 mM dithiothreitol (DTT), 50 mM piperazine, pH 6.3 for papain digestion. Aliquots were incubated at 37°C for 16 h with 1 ml plain agarose or 100 U of protease coupled to 1 ml agarose (Sigma Chemical Co, St Louis, MO). The digested preparations were dialyzed against TC buffer, diluted with IT-DME and assayed for estrocolyone-I activity.

Hexane, dichloromethane and acetone extraction of serum

5 ml CDHuS aliquots were acidified with 0.5 ml 10 N HCl and extracted with 5 ml hexane or dichloromethane; the solvent phase was evaporated and resuspended in 5 ml of 2 mg HSA/ml in IT-DME. 5 ml acetone cooled at −20°C were added dropwise to 5 ml CDHuS cooled at 4°C; after centrifugation (2000 g × 15 min) the precipitate was resuspended and dialyzed against TC buffer. The supernatant was evaporated in a Speed Vac concentrator (Savant Instruments, Long Island, NY) and resuspended in 2 mg/ml HSA in IT-DME.

Acid extraction of serum

To determine whether or not estrocolyone-I activity was due to chalone-like oligopeptides [22], CDHuS was dialyzed against 50 mM NH_4HCO_3, lyophilized, resuspended in water and extracted with 2 M acetic acid. Precipitated proteins were removed by centrifugation at 100,000 g and the supernatant was dialyzed

twice against 10 vol of 0.5 M acetic acid. These supernatants were dialyzed against TC buffer, brought to original volume and assayed for cell proliferation at concentrations of 5 and 10%.

Denaturation studies

The effect of urea and DTT on estrocolyone-I activity was studied as follows: (a) urea crystals (ultrapure grade, Schwarz-Mann Biotech, Cleveland, OH) were added to 10 ml of CDHuS to a final concentration of 6 M. Comparable aliquots were treated with (b) 10 mM DTT, (c) 100 mM DTT, (d) 6 M urea plus 10 mM DTT and (e) 6 M urea plus 100 mM DTT. After 2 h incubation at room temperature, these preparations were dialyzed twice at 4°C for 16 h against 10 vol of 25 mM BTP containing: (a) 6 M urea, (b) 10 mM DTT, (c) 100 mM DTT, (d) 6 M urea plus 10 mM DTT and (e) 6 M urea plus 100 mM DTT, respectively. Precipitated material was removed by centrifugation and each preparation was dialyzed against TC buffer.

Cell cycle analysis

Cells were harvested in 0.2% triton X-100 in Hanks' balanced salt solution containing 2 μg 4'-6-diamidino-2-phenylindol dihydrochloride/ml [23]. 10^4 cells were analyzed by flow cytometry with a 350/356 krypton laser [24].

HSA

Depending on the concentration range expected, two methods were used. Levels between 5 and 60 mg/ml were measured by the standard bromcresol green method [25]. Levels below 5 mg/ml were measured by a double antibody ELISA based on the assay described for fibronectin by Rennard *et al.* [26]. Briefly, plate coating was performed using 100 ng HSA/0.2 ml for 24 h at 4°C; simultaneously, the first antibody (rabbit anti-HSA 1:4000) was incubated with antigen in liquid phase. After washing the plates and saturating plastic binding sites with 0.5% gelatine, the HSA-first antibody mixture was placed into the coated wells, incubated for 2 h at 22°C; after washing, 0.2 ml of alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Sigma) (1:500) were added for 2 h, washed and incubated for 30 min with 0.2 ml of 1 mg/ml *p*-nitrophenyl phosphate; the reaction was stopped with 0.05 ml 3N NaOH and read at 405 nm. This indirect ELISA was very sensitive (10 ng/ml) and reproducible. Anti-HSA serum was obtained as described previously [27].

Sex hormone binding globulin (SHBG)

Purification was done by ligand affinity chromatography [28]; the column breakthrough was used as SHBG-free plasma.

Unless specified otherwise, all chemicals were purchased from Sigma; TGF-β was from Collaborative Research (Waltham, MA). Porcine insulin was a gift from the Eli Lilly Co. (Indianapolis, IN).

RESULTS

Effects of serumless medium, CDHuS and E_2 on proliferation

Cell proliferation curves revealed the following pattern: proliferation rates were maximal for the first 48 h regardless of the presence of E_2 or CDHuS and later, they decreased significantly only in CDHuS supplemented medium [Fig. 1(A)]. At 96 h, 95% of cells in CDHuS had a G_0/G_1 DNA content [Fig. 1(B), bottom panel] while in IT-DME with or without E_2 and CDHuS plus E_2 cells proliferated maximally; the DNA content distribution was 57% in G_0/G_1, 36% in S, and 7% in G_2 [Fig. 1(B), top panel].

Estrocolyone-I bioassay

The best estimate of proliferative activity is doubling time (t_d) measured during the exponential phase [29]. For practical reasons, a single time point assay was developed based on the evidence presented in Fig. 1. This assay measures the cell number achieved by an inoculum of 5×10^4 cells after 96 h of exposure to plasma fractions added to IT-DME both in the presence and absence of 30 pM E_2. A control for maximal proliferation yield is given by a similar cell inoculum grown in IT-DME. A standard curve of estrocolyone activity is simultaneously run using serial dilutions of CDHuS. One unit of estrocolyone-I activity is defined as the amount necessary to inhibit a population doubling of an inoculum of 5×10^4 cells/well in 1 ml of IT-DME after 96 h of exposure. Estrocolyone-I was measured as described above because cell proliferation is an exponential function; therefore, conditions had to be set to allow maximal proliferation rates in the absence of inhibitor as depicted in Fig. 1(A) (cells cultured in IT-DME proliferated maximally as did cells in CDHuS plus E_2). One estrocolyone-I unit is contained in 3 mg serum protein (5% serum). Estrocolyone-I content is measured by serial dilution; the assay measures the lowest dilution to show an activity equal to 1 U.

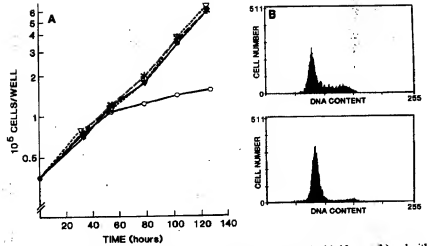

Fig. 1. (A) Cell proliferation curves of cloned MCF_7 cells grown in: IT-DME with (✱——✱) and without 30 pM E_2 (▽----▽); 10% CDHuS, both in the presence (●——●) and absence (○——○) of 30 pM E_2. Values indicate the mean of 4 experimental measurements; because SD was less than or equal in magnitude to the size of the plotted data point bars were not plotted. (B) Cell cycle profile by flow cytometry. Upper panel, cells in 10% CDHuS plus 30 pM E_2. Lower panel, cells in 10% CDHuS show a G_0/G_1 profile.

On the nature of estrocolyone-I activity

To assess whether or not estrocolyone-I is a small molecule, serum was extensively dialyzed against 100 mM NaCl, 25 nM HEPES, pH 7.4; membranes of nominal M_r cut-off ranging from 3.5 to 25 kDa retained estrocolyone-I activity (not shown). These results indicate that either estrocolyone-I is not a small molecule, or that it is tightly bound to a macromolecule. In addition, organic solvents such as acetone (Table 1), hexane and dichloromethane failed to extract estrocolyone-I activity from serum; this observation suggests that estrocolyone-I is not a steroid-like molecule.

Acid extraction of CDHuS was performed to assess whether or not estrocolyone-I activity was due to small chalone-like peptides; these peptides bind to macromolecules and are released from them by acid treatment [22]. Estrocolyone-I_c unlike chalones, was retained in the macromolecular compartment (Table 1).

Dialysis against 6 M urea, 25 mM HEPES, pH 7.4 also retained estrocolyone-I activity (not

Table 1. Characterization of estrocolyone-I: effect of serum components on the proliferation of MCF_7 cells

Source	Cell number[a]		E_2+/E_2- ratio	Control[b]
	Without E_2	0.3 nM E_2		
5% CDHuS[1]	130 ± 7	389 ± 31	3.0	375 ± 2
25 kDa membrane cutoff[1]	216 ± 4	482 ± 2	2.2	381 ± 18
6 M urea[1]	83 ± 2	217 ± 27	2.6	375 ± 2
6 M urea, 10 mM DTT[2]	370 ± 12	381 ± 18	1.0	375 ± 2
100 mM DTT[2]	215 ± 1	521 ± 5	2.4	520 ± 9
2 M acetic acid extract[2]	67 ± 2	160 ± 2	2.4	424 ± 13
Acetone extract[3]	533 ± 12	251 ± 8	1.1	310 ± 3
Acetone pellet[4]	125 ± 9	320 ± 8	2.6	310 ± 3
30 nM SHBG[5]	133 ± 8	165 ± 10	1.2	318 ± 3
5% SHBG-free plasma[6]	242 ± 8	472 ± 5	2.0	524 ± 5
2 mg HSA/ml[7]	361 ± 12	389 ± 3	1.1	341 ± 9

[a] Cell number is expressed as cells/well × 10^3 ± SD. [b] Control is the cell yield of a similar inoculum grown simultaneously in IT-DME. [1]: 5% CDHuS. [2]: CDHuS aliquots were treated as indicated; retained proteins were dialyzed against TC buffer and assayed at 3 mg/ml IT-DME. [3]: extracts were dried down and resuspended to the original serum volume with IT-DME containing 2 mg/ml HSA (Sigma, Cat No. A8763). [4]: pellet was resuspended to the original serum volume with TC buffer, dialyzed against TC buffer and diluted to 5% with IT-DME. [5]: SHBG was purified, molarity was calculated from its androgen binding capacity; after dialysis against DB, SHBG was diluted with IT-DME. [6]: The affinity column breakthrough was dialyzed against DB and diluted with IT-DME. [7]: Sigma, Cat No. A8763.

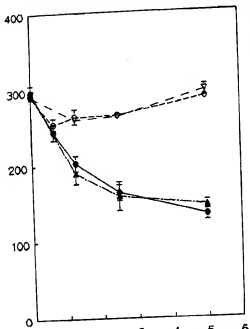

Fig. 2. MCF_7 cell yields were measured after 96 h of exposure to variable concentrations of: (i) CDHuS (normal donor) alone (●——●), or (ii) plus 30 pM E_2 (○——○), (iii) CDHuS (analbuminemic donor) alone (▲——▲), or (iv) plus 30 pM E_2 (▽——▽).

shown). Activity was not affected by dialysis against 10–100 mM DTT, 100 mM NaCl, 25 mM HEPES, pH 7.4; however, dialysis against 6 M urea, 10–100 mM DTT, 25 mM HEPES, pH 7.4 resulted in complete loss of estrocolyone-I activity (Table 1). These results are consistent with the notion that estrocolyone-I is a macromolecule; the inactivation effect of DTT only in the presence of 6 M urea suggests that a disulfide bond necessary for estrocolyone-I activity is either not accessible or it regenerates readily in non-denaturing conditions.

To assess whether or not proliferation inhibition by serum was due to trapping of estrogens by the serum proteins SHBG and HSA, these purified proteins were added to IT-DME. Neither inhibited MCF_7 cell proliferation; moreover, SHBG-free plasma obtained by adsorption of SHBG to cortisol-Sepharose retained the estrocolyone-I activity. TGF-β was marginally inhibitory at 0.1 nM but this effect was not cancelled by E_2. Cell yields were: $118{,}000 \pm 11{,}000$ in IT-DME; $86{,}000 \pm 7000$ in IT-DME + TGF-β and $72{,}000 \pm 4000$ in IT-DME + TGF-β + 0.3 mM E_2.

Estrocolyone-I activity in analbuminemic human serum

To ascertain whether or not estrocolyone-I activity is due to HSA as proposed by Laursen *et al.* [30], three strategies were implemented: (a) chromatographic separation as reported below, (b) testing the inhibitory activity of commercially obtained HSA (Table 1), and (c) testing the inhibitory activity of serum from an analbuminemic donor [31]. The HSA concentration in analbuminemic serum was <0.1 mg/ml, while in the normal serum used as control the HSA concentration was 40 mg/ml. Figure 2 compares the dose–response curve to normal and analbuminemic CD serum; both curves are superimposable indicating that estrocolyone-I activity is present in analbuminemic serum.

Protease treatment

Human serum contains macromolecular protease inhibitors such as α_1-anti-trypsin; this precludes the use of trypsin treatment of whole serum to assess whether or not estrocolyone-I is a trypsin-sensitive macromolecule. CD-avian serum also inhibits the proliferation of MCF_7 cells but, unlike human serum, it does not contain α_1-anti-trypsin. Trypsin digestion of rooster serum resulted in loss of inhibitory

Table 2. Protease sensitivity of serum estrocolyone-I activity

Treatment	Control: Cell number, No E_2	Control: Cell number, 0.3 nM E_2	Control: E_2+/E_2- ratio	Enzyme-treated: Cell number, No E_2	Enzyme-treated: Cell number, 0.3 nM E_2	Enzyme-treated: E_2+/E_2- ratio
Trypsin[a]	180 ± 8	355 ± 14	2.0	285 ± 16	324 ± 24	1.1
α-Chymo-trypsin[b]	91 ± 3	287 ± 76	3.2	174 ± 32	265 ± 7	1.5
Trypsyn[b]	114 ± 3	259 ± 13	2.3	168 ± 33	219 ± 8	1.3
Papain[b]	144 ± 2	395 ± 10	2.7	161 ± 3	168 ± 37	1.0

[a] CD-rooster serum was treated with 0.25 mg trypsin/ml, 2 h at 37°C; 5000 U soybean trypsin inhibitor/ml were added, and serum was assayed at 10%.

[b] 1 ml agarose (control) or immobilized enzyme (100 U/ml agarose) were incubated with 5 ml Maitrex-green fraction; after incubation for 16 h, at 37°C and dialysis samples were treated in culture at 10% dilution. Results are expressed as the mean ± SD of a set of three experiments.

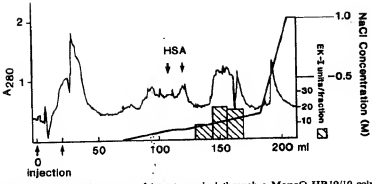

Fig. 3. 50 mg Matrex-green eluate were chromatographed through a MonoQ HR10/10 column. Start buffer: 20% acetonitrile, 2 mM DTT, 1 mM benzamidine, 50 mM piperazine, pH 6. Limit buffer: 1 M NaCl in start buffer.

activity (Table 2). Human plasma was treated with 80 mM $BaCl_2$ to remove endogenous proteases and chromatographed through Matrex-green agarose to remove endogenous protease inhibitors. The retained fraction containing 50% of the estrocolyone-I activity and most HSA was eluted with 2 M NaCl, 25 mM HEPES, pH 7.0, dialyzed, and digested with proteases covalently bound to the agarose gel as described in Methods. Protease-treated human estrocolyone-I fractions no longer inhibited MCF_7 cell proliferation (Table 2).

Separation of estrocolyone-I and HSA

Estrocolyone-I and HSA coprecipitated with $(NH_4)_2SO_4$ between 60 and 70% saturation. HSA and estrocolyone-I were retained by and coeluted from blue-Sepharose. Matrex-green also bound HSA; the breakthrough fraction contained only 15% of the HSA load with an estrocolyone-I yield of 50%. This fraction was used for further estrocolyone-I purification. A 1.5- to 3-fold purification was achieved by this procedure.

Ion exchange chromatography of the Matrex-green breakthrough

Estrocolyone-I was adsorbed to cation exchangers at pH 4.0 and to anion exchangers at pH 6.0. Chromatographic titration curves were performed on a MonoQ column; estrocolyone-I and HSA coeluted at pH 7.0 and 8.0 and were resolved at pH 6.0 and 9.5. Binding at pH 6.0 and adding 20% acetonitrile and 2 mM DTT to the start and limit buffers improved separation (Fig. 3). Estrocolyone-I activity was eluted between 120 and 170 mM NaCl. In a series of 3 experiments the purification achieved through this step was 30- to 40-fold; the overall purification was 40- to 60-fold. A representative experiment is shown in Table 3.

Hydrophobic interaction chromatography (HIC)

Complete estrocolyone-I adsorption was achieved at 2 M $(NH_4)_2SO_4$; estrocolyone-I was eluted with a linear gradient. This chromatographic step resulted in a 7- to 14-fold purification when performed after Matrex-

Table 3. Estrocolyone-I purification by anion exchange chromatography

Fraction	Protein content[a]	Specific activity[b]	Total yield[c]	Yield (%)[d]	Purification[e]
$BaCl_2$-plasma	155.00	1.33	199.0	100	1.0
Matrex-green breakthrough	80.00	2.00	100.0	50	1.5
MonoQ fraction No. 5	1.38	11.40	15.7	8	7.5
MonoQ fraction No. 6	0.25	80.00	20.0	10	60.0

Purification procedures are described in Methods. Anion exchange chromatography was performed in a MonoQ HR10/10 column; the start buffer was 20% acetonitrile, 2 mM DTT, 1 mM benzamidine, 50 mM piperazine, pH 6, and the limit buffer was 1 M NaCl in start buffer.

[a] Protein content is expressed in mg.

[b] Specific activity is expressed in estrocolyone-I U/mg protein.

[c] Total estrocolyone-I yield is calculated as the product of the protein content by the specific activity of the estrocolyone-I preparation.

[d] Percent yield is calculated as 100 × total estrocolyone yield of each purification step/estrocolyone-I yield of the starting material ($BaCl_2$-plasma).

[e] Purification is calculated as the ratio between the specific activity achieved after a purification step and the initial specific activity of the starting material.

Table 4. Estrocolyone-I purification by hydrophobic interaction chromatography

Fraction	Protein content (mg)	Specific activity[b]	Total yield[c]	Yield (%)[d]	Purification[e]
$BaCl_2$-plasma	110.00	1.00	110.0	100	1.0
Matrex-green breakthrough	31.96	2.20	70.3	63	2.2
2 M $(NH_4)_2SO_4$ supernatant	13.60	5.00	68.0	61	5.0
Phenyl Superose fraction No. 6	0.18	41.70	7.5	10	41.7
Phenyl Superose fraction No. 7	0.21	35.70	7.5	10	35.7

Purification procedures are described in Methods. Hydrophobic interaction chromatography was performed in a Phenyl Superose HR5/5 column; the start buffer was 2 M $(NH_4)_2SO_4$, 50 mM sodium phosphate pH 7 and the limit buffer was 20% acetonitrile, 50 mM sodium phosphate, pH 7.

[a] Protein content is expressed in mg.

[b] Specific activity is expressed in estrocolyone-I U/mg protein.

[c] Total estrocolyone-I yield is calculated as the product of the protein content by the specific activity of the estrocolyone-I preparation.

[d] Percent yield is calculated as 100 × total estrocolyone-I yield of each purification step/estrocolyone-I yield of the starting material ($BaCl_2$-plasma).

[e] Purification is calculated as the ratio between the specific activity achieved after a purification step and the initial specific activity of the starting material.

green chromatography; the overall purification achieved was 37- to 47-fold. A representative experiment is shown in Table 4. Phenyl Superose chromatography of the MonoQ estrocolyone-I fraction resulted in a further 10-fold purification (Fig. 4).

Estrocolyone-I isoelectric point by chromatofocusing

HSA-free estrocolyone-I obtained by MonoQ chromatography was chromatofocused using a 5.5 to 4.0 pH gradient. The inhibitory activity eluted at pH 4.5 to 4.8 (Fig. 5).

Estrocolyone-I molecular weight

Chromatography of CDHuS through a Superdex-200 column resulted in coelution of HSA and estrocolyone-I (not shown). Estrocolyone-I purified by anion exchange chromatography eluted from a Superose 12 column at volumes corresponding to a M_w of 70–90 kDa (Fig. 6). Comparable molecular weight was obtained using 6 M urea in the running buffer.

DISCUSSION

We described the inhibitory effect of serum on the proliferation of both human and rodent estrogen sensitive cells [10, 11, 14–16]. Estrogens cancel this inhibitory effect by mechanisms not fully understood [16]. Herein, we describe physicochemical properties of the molecule putatively responsible for the inhibitory effect of serum, namely, estrocolyone-I. The proliferation–inhibiting activity remained in the macromolecular compartment after extensive dialysis against 0.5 M acetic acid through membranes of a nominal cutoff = 25 kDa and was not extracted by organic solvents (Table 1). Protease treatment of unfractionated mammalian serum was hindered by endogenous protease inhibitors; removal of protease inhibitors from human

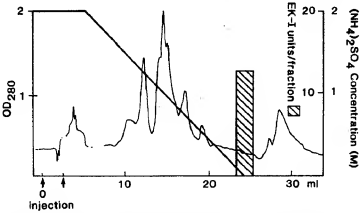

Fig. 4. 10 mg MonoQ fraction were chromatographed through a phenyl Superose HR5/5 column. Start buffer: 2 M $(NH_4)_2SO_4$, 50 mM sodium phosphate pH 7. Limit buffer: 20% acetonitrile, 50 mM sodium phosphate, pH 7.

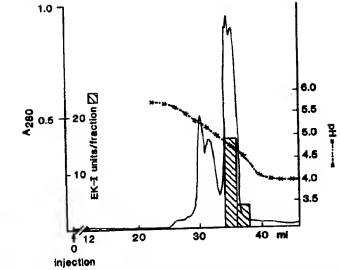

Fig. 5. Chromatofocusing profile of the MonoQ fraction. 25 mg protein were injected into a MonoP HR25/5 column equilibrated with 50 mM piperazine pH 5.5. After isocratic elution with this buffer, separation was achieved with a self generated pH 5.5–4 gradient by 10% Polybuffer pH 4.

plasma by Matrex-green chromatography followed by protease treatment completely abolished the inhibitory activity (Table 2). Trypsin treatment of chicken serum, which lacks anti-trypsin activity, significantly reduced estrocolyone-I activity. Reduction of disulfide bonds by DTT destroyed estrocolyone-I activity only when performed in the presence of denaturing concentrations of urea, suggesting that either critical disulfide bonds are not accessible in the native conformation or that they regenerate correctly only in this state. These results strongly suggest that estrocolyone-I is not a TGF-β-like molecule, since TGF-β activity is irreversibly destroyed by 10–100 mM DTT. Moreover, TGF-β did not significantly inhibit the proliferation of MCF_7 cells. This is an agreement with the results reported by Laursen *et al.* [30]. Acid extraction was performed to determine whether or not estrocolyone-I was a chalone-like oligopeptide; these experiments confirmed the macromolecular nature of estrocolyone-I activity. These data suggest that estrocolyone-I is a polypeptide; henceforth, protein purification methods were used for estrocolyone-I characterization.

$(NH_4)_2SO_4$ precipitation is often used to reduce the contaminant burden to the following chromatographic step. Estrocolyone-I and HSA coprecipitated with $(NH_4)_2SO_4$; because HSA is the most abundant plasma protein, this fractionation procedure was ruled out. These two moieties also cocluted by Cibacron blue chromatography. To determine whether or not the inhibitory activity of serum was due to albumin [30], the inhibitory activity of HSA

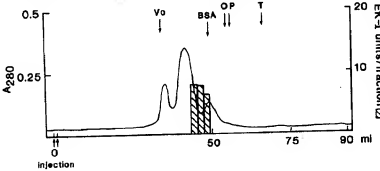

Fig. 6. Gel filtration profile of the MonoQ fraction. 30 mg protein (2 ml) were injected into a Superose 12 HR16/50 equilibrated with 100 mM NaCl, 50 mM BTP pH 7. Arrows indicate the elution of molecular weight markers. V_0: void volume (blue dextran), BSA: bovine serum albumin (66 kDa), O: ovalbumin (45 kDa), P: pepsin (34.7 kDa) and T: trypsinogen (24 kDa).

(Table 1) and of serum from an analbuminemic donor were assessed (Fig. 2). These results indicated that estrocolyone-I activity was not due to HSA.

HSA was removed by adsorption to Matrex-green. Estrocolyone-I isoelectric point was 4.5–4.8; the native molecular weight was 70–80 kDa. Both results were obtained using chromatographic techniques; since estrocolyone-I detection relies on a bioassay, the use of analytical gel electrophoresis and isoelectric focusing is precluded. Partial purification was obtained by HIC and anion exchange chromatography of the Matrex-green fraction. Substantial purification was achieved by using Matrex-green to remove HSA, followed by anion exchange chromatography and HIC. These results suggest that (1) estrocolyone-I is a protein, and (2) its inhibitory effect is not due to mere estrogen trapping since SHBG and albumin fail to inhibit MCF_7 cell proliferation (Table 1).

Finally, these data strengthen the notion that a plasma-borne factor, estrocolyone-I, is responsible for the serum-induced inhibition of the proliferation of estrogen-sensitive MCF_7 cells. Further purification of estrocolyone-I is being pursued, a step necessary for a mechanistic understanding of its action and that of estrogens on the control of cell proliferation.

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